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Void exclusion of antibodies by grafted-ligand porous particle anion exchangers

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ABSTRACT

We describe a new variant of anion exchange chromatography in columns packed with porous particles that embody charged low-density polymer zones supported by a higher density polymer skeleton. IgG defies the norms of anion exchange and is excluded to the void volume at pH 3–10 and 0–4 M NaCl. Void exclusion also occurs with Fab, F(ab')$_2$, and IgM. Host cell protein contaminants mostly follow the usual norms of anion exchange and bind more strongly with increasing pH and decreasing conductivity. Sample buffer composition has no impact on partitioning so long as applied sample volume does not exceed the interparticle void volume of the column. Void-excluded antibody elutes in equilibration buffer. This seemingly conflicted collection of behaviors is reconciled by a variable size exclusion function mediated through the low-density polymer zones, the charge properties of the antibody species, and the pH and conductivity of the equilibration buffer. Current-generation porous particle anion exchangers that employ grafting techniques to achieve high charge density mediate void exclusion to varying degrees, with the best-suited achieving complete exclusion, and others as little as 65%. Perfusive and non-grafted particle-based exchangers mediate as little as 50% exclusion. Monoliths mediate no exclusion, due to their lack of an interparticle void volume. On qualified exchangers, the technique supports greater than 99% reduction of host proteins, DNA, and endotoxin. Virus is reduced more than 99.9%, and aggregates are reduced to less than 0.05%. The method supports better process control than other anion exchange formats because pH excursions in conjunction with changes in salt concentration do not occur until after the antibody has eluted from the column.

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1. Introduction

Anion exchange chromatography has proven to be one of the most powerful and versatile techniques in the field of protein fractionation, and continues to be a center for innovation. This is especially true in the field of IgG purification where anion exchangers have diversified into porous particle, perfusive particle, membrane and monolithic formats embodying a wide range of ligands, which are further subdivided into various non-grafted and grafted configurations. Anion exchange is also the most universal technique in IgG purification, being the only method used more widely than biological affinity chromatography [1]. It is most commonly employed in so-called flow-through mode, where samples many times the volume of the exchanger are applied. The antibody elutes minus contaminants that bind. Most applications are based on an assumption that antibodies follow the usual norms of anion exchange, and are more likely to bind with increasing pH and decreasing salt concentration. Some investigators recommend conditions where the antibody partially binds, with the rationale that the sacrifice in recovery is rewarded with more effective contaminant removal and the potential for reducing the number of purification steps [2].

Research groups led by Zydney, by van Reis, and others have particularly highlighted the role of electrostatic repulsion between solutes and charged groups on ultrafiltration membranes [3–14]. The historical roots and current state of this application sector were reviewed recently by Rohani and Zydney [11]. Electrostatic repulsion of IgG from positively charged ultrafiltration membranes is the basis of a technique now called high performance tangential flow filtration (HPTFF) [11–14]. Charge repulsion prevents passage of IgG through 100–300 kDa membrane pores while neutral contaminants pass and acidic contaminants bind. Electrostatic repulsion is also the basis for ion exclusion chromatography, where it is exploited for fractionation of organic acids, sugars, and other small molecules [15–18]. Selectivity in all these systems obeys the expected rules of pH and conductivity [4,8,11,15–18]. Increasing like-charge increases repellency; increasing conductivity suppresses repellency.

It seems evident that electrostatic repulsion must also contribute to IgG behavior in other anion exchange formats, and that if it can be manipulated with ultrafiltration membranes, it

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should be possible to exploit it effectively in a fixed bed chromatography format. This study shows that this expectation is met only partially or not at all, except for two examples of a special class of anion exchangers that support highly effective antibody purification in a manner that defies the most fundamental assumptions of ion exchange while substantially extending its capabilities.

2. Materials and methods

Buffers, salts, and reagents were obtained from Sigma–Aldrich (St. Louis, MO). A rat IgM hybridoma, clone 60A9, was produced in 5% fetal bovine serum by SP2/0 cells. Chimeric and humanized biosimilar IgG monoclonal antibodies were produced in protein-free media by Chinese hamster ovary cells, employing a tricistronic vector described in [19]. Rituximab $F(ab')_2$ and Fab were prepared by digestion with immobilized enzymes as described in [20].

UNOsphere™ Q, Nuvia™ Q, and ceramic hydroxyapatite CHT™ type I 40 μm were purchased from Bio-Rad Laboratories (Hercules, CA). GigaCap™ Q was purchased from Tosoh Bioscience (Tokyo, Japan). Fractogel DEAE Hi-Cap was purchased from Merck (Darmstadt, Germany). Q Sepharose™ Fast Flow, Capto™ Q, Capto adhere, MabSelect™ (protein A) and Sephadex™ G25 were purchased from GE Healthcare (Uppsala, Sweden). POROS® HQ-50 and XS were obtained from Applied Biosystems (Foster City, CA). An 8 mL CIM™ QA monolith was obtained from BIA Separations (Klagenfurt, Austria). ProSep™-Va (protein A) was purchased from Millipore (Billerica, MA).

Particle-based media for anion exchange were packed in XK™16/20 columns (20 mL, 10 cm bed height; GE Healthcare) at a linear flow rate of 300 cm/h. The adaptor was brought to the surface of the bed with care to avoid axial compression. Samples were applied strictly and exclusively through a superloop to minimize pre-column dispersion at the sample boundaries. Protein A media were packed in XK or Tricorn™ series columns (GE Healthcare). Experiments were run at 200 cm/h. Column packing and chromatography experiments were conducted on an AKTA™ Explorer 100 (GE Healthcare).

In some anion exchange experiments, 500 mM NaCl was added to the sample to highlight the relative distribution of antibodies and salts. Experiments run at pH 3.0 were buffered with 50 mM citrate; at pH 4.0 with 50 mM acetate; at pH 5 with 25 mM acetate, 25 mM MES; at pH 6.0 with 50 mM MES; at pH 7.0 with 50 mM Hepes; at pH 8.0 with 50 mM Tris; at pH 9.0 and 10.0 with 50 mM borate. Purified IgG was prepared by applying filtered cell culture supernatant (CCS) to protein A affinity media equilibrated with 50 mM phosphate, 100 mM NaCl, pH 7.2. The column was washed after loading with equilibration buffer, then eluted with 100 mM arginine, 100 mM acetate, pH 3.5 (MabSelect) or 100 mM citrate, pH 3.0 (ProSep). In one experiment, initial purification of trastuzumab was conducted by cation exchange chromatography on POROS HSX. CCS titrated to pH 6.0 was diluted to a conductivity of 2.5 mS/cm, and loaded onto a column equilibrated with 50 mM MES, pH 6.0. This was followed by washing with equilibration buffer, then elution in a linear gradient to 50 mM MES, 200 mM NaCl, pH 6.0.

The 8 mL anion exchange (QA) monolith was evaluated by equilibrating it to 50 mM Tris, pH 8.0, and loading a 2 mL sample of purified biosimilar Trastuzumab™ through a superloop, at a flow rate of 50 mL/min.

In one experiment, contaminating free light chain and light chain dimers remaining in trastuzumab after anion exchange processing of CCS were removed by cation exchange chromatography as described above. In another experiment, light chain contaminants were removed by binding the sample to Capto adhere at 1 M NaCl, 50 mM Hepes, pH 7.0, then eluting with a descending linear gradient to 50 mM Hepes, pH 7.0. In another experiment, light chain contaminants were removed by binding the sample to hydroxyapatite in 50 mM Hepes, 5 mM sodium phosphate, pH 7.0, then eluting with a linear gradient to 1 M NaCl, 5 mM phosphate, pH 7.0, prior to cleaning with 500 mM phosphate, pH 7.0.

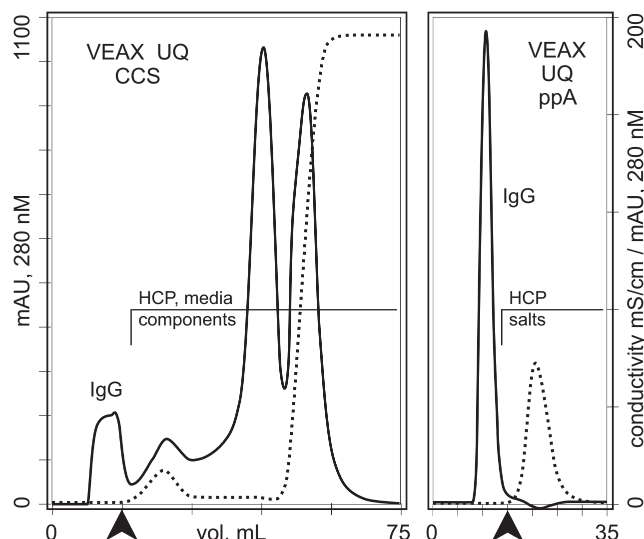

Fig. 1. Chromatograms of filtered CCS and protein A-purified trastuzumab applied to UNOsphere Q. Sample volume 35% of column volume. Both columns equilibrated to 50 mM Tris, pH 8.0. Solid line: UV absorbance. Broken line: conductivity.

Antibody aggregate content was monitored by size exclusion chromatography (SEC) on a Shimadzu HPLC system (Kyoto) with a G3000SWxl column (Tosoh Bioscience) equilibrated with 50 mM MES, 200 mM arginine, 5 mM EDTA, 0.05% sodium azide, pH 6.5) at 0.6 mL/min. 100 μL of sample was injected. Host cell proteins (HCP) were monitored by ELISA according to the manufacturer's recommendations with a CHO HCP kit from Cygnus Technologies Inc. (Southport, NC). Host Cell DNA was measured by intercalating dye assay according to manufacturer's recommendations with the AccuBlue™ High Sensitivity dsDNA Quantitation kit (Biotium, Inc., Hayward, CA). Endotoxin levels were measured by a standard kinetic chromogenic Limulus Amebocyte Lysate assay using Endosafe Endochrome-K™ LAL reagent (Charles River Laboratories Inc., Wilmington, MA). Infectivity testing for minute virus of mice (MVM) and murine leukemia virus (MuLV) was conducted by Charles River Laboratories (Cologne, Germany).

Other experimental details are described in the following section.

3. Results and discussion

During the course of routine flow-through anion exchange chromatography of biosimilar trastuzumab (IgG) on UNOsphere Q, we observed a well-defined shoulder on the leading edge of the breakthrough fraction. Conductivity and pH of the shoulder matched the column equilibration conditions. Given the operating pH of 7.0 and the antibody isoelectric point (pI) of 8.45 [21], we assumed that this represented the antibody being repelled from the exchanger surface and forced to migrate exclusively through the void volume. When the column was loaded to 35% or less of its total volume, a single population of highly purified antibody eluted entirely within the void, with good separation from the majority of contaminants (Fig. 1).

We anticipated that increasing salt concentration of the equilibration buffer would reduce or eliminate repulsion, allow IgG to diffuse into particle pores and suspend its exclusion. Peak width increased incrementally with NaCl concentration up to 4 M,

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